# STAIN TECHNOLOGY

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## PROGRESS IN THE STANDARDIZATION OF STAINS

### DYES FOR THE EOSIN-METHYLENE BLUE MEDIUM

A well-known medium among bacteriologists is Levine's eosin-methylene blue agar for the differentiation of the colon-typhoid group of bacteria. Reference to this use of the dyes has been made in Biological Stains and in other publications of the Stain Commission, but until this spring no samples of methylene blue or eosin have been certified for this purpose.

The reason for this is that by no means all samples of these two dyes are satisfactory for this medium, and it is a time-consuming task to determine whether or not any given sample can be so used. There are so many possibilities in the way of combining the two dyes in a medium that the Stain Commission has found it almost impossible to undertake the tests necessary to determine whether any sample submitted is satisfactory or unsatisfactory for this purpose.

Recently, however, one company has submitted samples of these two dyes intended especially for this purpose, and has done the laborious part of the work before applying for certification. The two dye samples were sent to us with exact instructions as to how much of each to employ in the medium, thus making the matter of testing them a very simple one. Under these conditions we gladly tested the samples, and have found them both satisfactory for the purpose for which they were intended.

The company submitting these samples is not regularly in the stain business and does not pretend to manufacture the dyes. They have been putting out the eosin-methylene blue agar in dehydrated form for a number of years, and the dyes which they plan to sell with our certification for this purpose are the same ones which they will use in the dehydrated medium in the future. In this way bacteriologists who prefer to make up their own medium can purchase the dyes from this company and profit by the company's experience in selecting satisfactory dyes for the purpose. An advertisement of this grade of methylene blue and eosin appears elsewhere in this issue.

—H. J. CONN

## Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the April number of this journal.

Stains Certified March 1, 1933, to May 31, 1933*

| Name of dye | Certification No. of batch | Dye Content | Objects of tests made by Commission† | Date approved |
|---|---|---|---|---|
| Crystal violet | CC 6 | 85% | As bacteriological, histological and cytological stain | Mar. 17, 1933 |
| Basic fuchsin | NF 22 | 81% | As bacteriological stain, and for use in Endo medium | Mar. 18, 1933 |
| Methyl violet | NMv 4 | 81% | As histological and cytological stain | Apr. 4, 1933 |
| Fast green FCF | NGf 2 | 94% | As histological counterstain | Apr. 4, 1933 |
| Acid fuchsin | CR 5 | 75% | As histological stain, and for use in Andrade indicator | May 20, 1933 |
| Eosin Y | DE 2 | 93% | As constituent of Levine eosin-methylene-blue agar | May 23, 1933 |
| Methylene blue | DA 2 | 84% | As constituent of Levine eosin - methylene - blue agar | May 23, 1933 |

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

# JOHN BELLING

October 7, 1866—February 16, 1933

ALBERT F. BLAKESLEE, *Dept. of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, N. Y.*

In the death of Dr. John Belling of anemia in his sixty-seventh year, the science of biological staining has lost a painstaking and brilliant investigator.

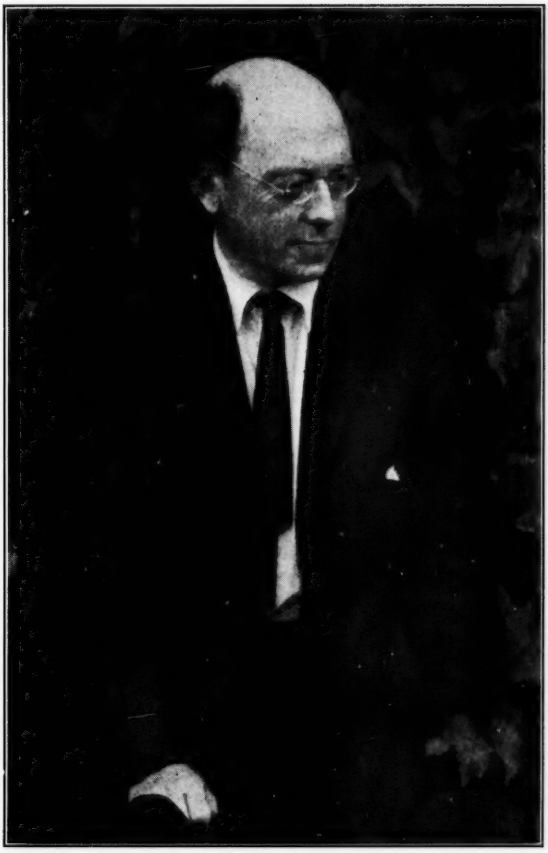
He was born n Aldershot, England, the son of an army school master, studied in the University of Birmingham and received the degree of B.Sc. from the University of London in 1894. In 1922 he was given the honorary degree of D.Sc. from the University of Maine. After teaching in various institutions in England, he was teacher and investigator in the Imperial Department of Agriculture of the British West Indies from 1901 to 1907. From 1907 till 1916 he carried on botanical research for the Florida Experiment Station. It was here that he made his classical studies of semisterility in hybrid velvet beans (*Stizolobium*) which a dozen years later found its final explanation in his hypothesis of segmental interchange of chromosomes. In 1921 he came to Cold Spring Harbor to the Department of Genetics of the Carnegie Institution of Washington where he began his intensive study of chromosomes and their behavior which was continued thruout the rest of his life. It was here that he perfected the aceto-carmin method of staining by the addition of iron which rendered a clear differentiation between chromosomes and cytoplasm possible. What a single method in stain technic may mean to the development of a science would be realized if a moratorium for a year should be declared on Belling's iron-aceto-carmin method and its various later modifications. The writer of this note realizes that such a moratorium would put the cytological work on *Datura* temporarily out of business.

Dr. Belling's contributions were far from being along lines of technic alone. At Cold Spring Harbor, with the aid of his iron-aceto-carmin method and optical refinements, he studied chromosomes of a considerable number of plant species including *Canna*, *Cypripedium*, *Hemerocallis*, *Hyacinthus* and *Uvularia*. As cytologist in the *Datura* investigations, which were one of his chief interests during this period, he gave the final answer to many problems which the breeding behavior had raised and predicted genetic results from the chromosomes as he saw them in the microscope. His basal contribution, from which other hypotheses such as segmental interchange

were developed, was his realization that the arrangement of chromosomes into pairs at reduction is due to the organization within the individual members of a pair such that like ends are attached to each other at this stage of division. The attachments were used by him as a method of identifying specific ends of chromosomes in groups of more than two. He was thus able to give cytological evidence for the specificity of the extra chromosome in chromosomal types at a time when it is believed by some of the leading investigators of such types in *Oenothera* that the extra chromosome is a characteristic rather than a cause of a given mutant type and that it is a matter of indifference to the plant which chromosome is extra. His influence in changing cytological concepts can be further realized when it is remembered that less than a half dozen years ago the current belief held that the attachment of chromosomes into circles of several members, so common in *Oenothera*, is due to a general affinity between maternal and paternal chromosomes or that circle formation is a genetic character inherited like any other genetic character due to a gene.

Dr. Belling was the first, at least among botanists, who in his thinking utilized to the full measure both the genetic and the cytological viewpoints. He had unusual mathematical ability and a grasp of chemistry and physics which he applied to the perfection of the technic necessary to his investigations. His thoro understanding of optics is shown by his book on the microscope. This tool of research he used to its full optical capacity. As an example may be mentioned that, finding there were three reflections from the ordinary silvered mirror on a microscope, he cemented a right-angled reflecting prism in its place which gave only a single reflection and therefore afforded better definition. This prism mirror he had been using for some time before he came across a reference in the literature to a similar suggestion.

In the University of California, where he carried on investigations for the Carnegie Institution of Washington from 1927 until the time of his death, he perfected the iron-brazilin method with which he investigated the chromosomes of liliaceous plants. Under the best conditions of fixation and staining and with refined optical equipment, he was able to see the finer details of chromosomal structure as never before, and in the ultimate chromomeres he believed he had seen and identified the very genes themselves. Until recently, little has been known cytologically about the chromosomes in the earlier stages of the reduction divisions where genetic evidence indicates crossing over must take place. It was just these stages that were being in-



JOHN BELLING, 1866–1933

vestigated by him in his last days. Knowing his skill in technic and observation, and his ability in formulating hypotheses with a strong factual foundation, together with a willingness to revise his conclusions to conform with new evidence, we had looked to Belling to lead the way in the solution of these difficult and, at present, somewhat controversial problems. His death is a great loss to the newly developing science of cytogenetics.

# METHODS FOR THE STANDARDIZATION OF BIOLOGICAL STAINS[1]

## PART I

A. R. PETERSON,[2] H. J. CONN, and C. G. MELIN[3]

*Contribution No. 223 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Dept. of Agric., Washington, D. C.*

## GENERAL CONSIDERATIONS

ABSTRACT.—**This is the introduction to a series of papers giving the assay methods employed in the testing of dyes submitted to the Commission on Standardization of Biological Stains. These methods are used in determining whether or not to allow the sale of any particular batch of dye as a certified stain. The present paper takes up general considerations, discussing the relative merits of various analytical methods designed both for qualitative and quantitative analysis. The advantages of determining light "absorption ratios" for qualitative purposes, and of titanous chloride reduction for quantitative purposes are pointed out. It is further indicated that in spite of all the refinements that have yet been made in chemical and optical methods of analysis, great weight must be placed on biological testing in determining the quality of any given sample.**

With the increasing and diversified use of dyes in biological work, the necessity for fully sufficient methods for their analysis is increasing. Much improvement has already resulted in biological staining from the preliminary work which has been carried out to determine the chemical nature and specific application of many biological stains.

The chemistry and analysis of the permitted food dyes has been discussed by Ambler, *et al.* (1928) in considerable detail,[4] but similar definite information is available on only a comparatively small number of biological stains. Such general information on the subject as is of value is widely scattered thruout the literature. In view of this

[1]The authors are deeply indebted to W. C. Holmes, late Senior Chemist of the Color and Farm Waste Division of the Bureau of Chemistry and Soils, and to J. T. Scanlon, Associate Chemist of the same Division, for their development of many of the analytical methods given in this paper.

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[4]For the identification of color substances in foods, see Allen's Commercial Organic Analysis, **5**, 425–508, Edition 5. 1927.

fact, it has been deemed advisable to put in usable form the methods now employed in the standardization of biological stains for certification by the Biological Stain Commission.

Before considering individual procedures[5], it is believed that a short discussion of the merits and limitations of available methods in general will be useful to the analyst by indicating the most promising methods for the attack of his problems.

## Analytical Methods

*Qualitative Analysis:* The qualitative examination of dyes is a prerequisite to their quantitative analysis. The earliest attempts at dye identification were along chemical lines. The first definite scheme of analysis was proposed by Witt (1886). Alternate schemes were proposed subsequently by Weingärtner (1887), Green (1893), and Rota (1898). Green later (1914) published his revised and improved methods in a book which has become the standard work on the subject. The system employed is of excellent service in determining the general type of dye under investigation, and such work as has since been carried out on the identification of dyes by chemical means has been largely a mere amplification of Green's methods.

Altho the value of Green's contributions to dye analysis should be acknowledged, it is necessary to emphasize the limitations of his scheme. With the possible exception of the azo dyes, the system employed by Green, even with the addition of further "color reactions" as cited by Schultz (1928–31) and the Colour Index (Rowe, 1924), and carried out with refinements in technic (Holmes and Berliner, 1927), frequently fails to differentiate between different dyes of the same type.

In the field of identification of azo dyes, it may prove that chemical methods are as reliable (altho not as convenient) as physical means. Such dyes are readily split by either oxidizing or reducing agents, the scission occurring in either instance at the azo linkage, and the identification of the reaction products may be made to serve in identifying the dye. A comprehensive list of the reduction products of azo dyes, together with such of their properties and reactions as might be of service for their identification, has been made by Holmes (1925). Since the publication of those data, the results have been reported

[5]The individual methods in the papers of this series that are to follow are those now in use by the Commission on Standardization of Biological Stains, in cooperation with the Color and Farm Waste Division of the Bureau of Chemistry and Soils of the Dept. of Agric. These methods of analysis, it must be understood, merely represent those that are at present in use; they are not regarded as the best possible procedures, and are subject to revision as more refined or simpler ones are developed.

from three extensive investigations, bearing on the identification of azo dyes, by Forster and Hansen (1926), Fierz-David (1926) and Brunner (1929).

With dyes in general, absorption spectra[6] are usually highly characteristic and chemical methods of differentiation relatively ineffective, so there is little or no question but that spectroscopic or spectrophotometric methods of identification are greatly to be preferred. They are rapid and convenient and may be applied with extremely minute amounts of material.

Formánek, *et al.* (1908–27) have worked out a suitable scheme for the spectroscopic identification of dyes and have published absorption maxima and other information on individual dyes. In identifying a dye, the analyst determines first the subdivision in which it will be found in Formánek's scheme. He then determines its absorption maximum under a variety of conditions which were employed by Formánek in obtaining data with dyes of that type. If his values are in close agreement with those which Formánek has recorded for some specific dye, the analyst may be reasonably sure that his sample is identical with the dye in question.

The objections which may be made to the Formánek method are that it relies almost entirely on precise determination of the location of absorption maxima, and that differences in this particular are frequently too small in closely related dyes to afford a thoroly satisfactory means of differentiation. Also it is a matter of some little difficulty to determine dye maxima with any considerable precision, as the results may be affected by the type of instrument used and by the technic of the analyst.

The difficulty experienced in locating definite absorption maxima led to the development of an alternate practice. Wave lengths were selected on opposite slopes of the absorption bands of the dyes in suitable solutions, and the ratios of the extinction coefficients determined by means of the spectrophotometer. These "absorption ratios" serve substantially the same purpose as do the band maxima, in that they serve to locate the spectral positions of the dye bands. Tested with the various basic violets of the triphenylmethane group, Holmes (1925), found that the ratios provided a much better means of distinguishing between similar dyes than did the dye maxima. In a later investigation, Holmes and Peterson (1930) tested the usefulness of

[6]Color arises from the selective absorption of light in the visible spectrum. The color of a dye is determined by the relative degree in which it absorbs light in different portions of the spectrum. The manner in which a dye absorbs light is termed its absorption spectrum.

absorption ratios for purposes of identification with about 90 dyes which find application in biological staining. The accuracy with which the ratios could be determined varied, as would be expected, depending upon relative visual sensitivity within the regions wherein it was necessary to make the measurements. The authors concluded that absorption ratios were less satisfactory with yellow, orange, or brown dyes than with dyes of other colors, not only because of inferior visual sensitivity within the region of measurements, but also because it was usually necessary to make both measurements on one side of the maximum absorption. They afforded a very reliable means, however, for differentiating between similar dyes of other colors.

Commercial stains commonly contain varying proportions of subsidiary coloring matters which have decidedly different absorption ratios from that of the dye with which they are associated. It would be expected, accordingly, that a given dye would not be found to have a perfectly definite and invariable absorption ratio, but rather that its ratio would vary in accordance with the nature and proportion of colored impurities present.

This expectation was realized. Some dyes proved substantially uniform, but others gave some diversity in values for different samples. It appears improbable, however, that this circumstance will militate seriously against the utility of absorption ratios for practical purposes of identification, as the differences in ratio values of different dyes are so considerable, in general, and the effects of moderate proportions of color impurities so small in comparison, that the identity of the dye will seldom be obscured. The analyst will recognize abnormality in samples, as he may encounter it.

*Quantitative Analysis:* Altho commercial samples of stains contain moisture and inorganic salts or other impurities, the principal, and ordinarily the only, demand made upon the dye analyst at present is that of determining "actual dye content". The ultimate analysis of dyes may be undertaken by the methods which are applied with organic substances in general, but this is seldom done in routine analysis. With many dye samples which are marketed in admixture with dextrin, it would prove entirely worthless, while with stains in general more convenient methods are desired. It is often necessary, however, to standardize or at least to test the more rapid and convenient analytical methods with dyes of known purity; and ultimate analysis may afford the best or only means available for establishing the purity of the dyes employed for such purposes.

Altho a variety of different methods have been proposed for the quantitative analysis of dyes, only two methods may safely be employed extensively. Chemical reduction with standard solutions of

reducing agents and spectrophotometric evaluation give relatively reliable results in general, and both are applicable with the great majority of biological stains. Other methods are less universally applicable and, as shown by Holmes and Peterson (1930), are liable to prove misleading when employed with such inferior grades of dyes as are frequently obtained on the market.

The principles of quantitative spectrophotometry are simple, as explained by Krüss and Krüss (1891). Absorption bands of dyes vary in both location and intensity with change in solvents. With any given solvent, however, under given suitable conditions, the extinction coefficients of dye solutions vary in direct proportion with their dye concentrations. In other words, dye solutions obey Beer's law (1852) under the proper conditions. Having once obtained absorption data (extinction coefficients) on a solution of a given dye of known concentration under regulated conditions, the dye contents of all future samples of that dye may be determined readily on the basis of their comparative absorptions when examined under the same conditions. A judicious technic is essential if appreciable errors are to be avoided, as all factors which affect either the chemical or the physical nature of the dye molecule require consideration. All these factors have been discussed in detail elsewhere (Holmes, *et al*, 1932).

The evaluation of dye samples is generally made by means of reduction with titanous chloride in accordance with the principles outlined in Knecht and Hibbert's monograph (1925).

The titanous chloride solution is prepared with excess hydrochloric acid and stored under hydrogen.[7] Under such conditions it is essentially stable, requiring standardization only at considerable intervals. In its employment in the titration of dyes, a current of carbon dioxide is used to preclude all possibility of extraneous atmospheric oxidation. The dye solution is usually brought to a boil before reduction and titrated hot. Complete reduction is evidenced by the disappearance of color or by a definite color change in this lightly colored reduced solution. It is usually possible to titrate dyes by the direct method, but it may prove advantageous and in some cases necessary to introduce an excess of titanous chloride and determine the residual reducing agent subsequently by appropriate means. In instances where the visual end point is difficult, resort may be had to electrometric technic by the procedure of Jones and Lee (1922). In this method, the dye is dissolved in a small amount of water and sulfuric acid

[7]The method of preparation, storage, standardization and use of the titanous chloride solution has been given in detail by F. L. English in J. Ind. Eng. Chem., **12**, 994, 1920, and in Dept. Bul. No. 1390 of the U. S. Dept. of Agric.

added, instead of hydrochloric, as Knecht and Hibbert (1925) recommend. The titration flask is then swept with carbon dioxide, titanous chloride added in slight excess, and the excess titrated back with ferric alum. The end point is detected by the deflection of a galvanometer. The error in this method is very small.

Dyes are usually titrated in aqueous solution, but it is frequently of advantage, as shown by Calcott and English (1923) to modify the solvent by the addition of alcohol or acetic acid to improve the solubility of the dye or its reduction products. The nature of the medium in which the dye is dissolved affects considerably the difficulties encountered due to high coloration of the reduced solution which masks the end point of the titration. With the majority of dyes it was found by Evenson, *et al.* (1928, 1931) that the addition of tartrates or other organic salts, which function as buffers in limiting the degree of acidity in the solution and also appear to exert a catalytic influence on the reduction, will effect a more accurate evaluation of color in the dye samples. Insoluble dyes may be titrated if they are first converted into a soluble form by means of sulfonation.

Most biological stains are completely reduced without difficulty. The formation of the leuco derivatives of the dyes of the triphenylmethane and quinonimide groups require four hydrogen equivalents; nitroso groups require four; and nitro groups six. The theoretical titanous chloride consumption may be calculated for any dye of known constitution and, conversely, when the identity of the dye is known, its dye content may be calculated from the observed consumption of titanous chloride. The accuracy of the method is excellent when the technic followed is varied to conform with the optimum conditions for the individual dyes under examination. The titanous chloride method is not universally applicable but has a far wider range of utility than any other chemical method available.

## Biological Tests

It has not ordinarily been possible to depend solely on the chemical or spectrophotometric examination of stains as a means of their standardization in biological use. Too many factors that are still unknown enter into the situation and affect the behavior of the dyes. It has frequently been found, for instance, that two different samples of basic fuchsin, identical in dye content and absorption spectra, might vary decidedly in their staining properties or their behavior in other biological applications. Safranin is another instance where the same situation has occurred.

When the standardization of stains was first undertaken, so little was known about the correlation of their chemical with their biological behavior that instances such as the two dyes just mentioned were the general rule. At the present time, altho there are others besides these two dyes where complete dependence cannot be placed on chemical or optimetric tests, the number of such cases is steadily being reduced.

Even up to the present time, however, it is felt necessary to test every batch of stain in its behavior for the uses for which biologists regularly employ it, as a supplement to the chemical and physical tests. As a result of this, a series of biological tests has been drawn up for the various stains which are now undertaken in a routine way parallel with the chemical and optimetric tests.

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# METHODS FOR THE STANDARDIZATION OF BIOLOGICAL STAINS

## PART II

A. R. Peterson,[1] H. J. Conn, and C. G. Melin[2]

*Contribution No. 225 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Dept. of Agric., Washington, D. C.*

## THE FLUORANE DERIVATIVES

Abstract.—**In this paper the methods are given which are used in determining whether to approve the sale of certain dyes of the fluorane group as certified biological stains. The methods have been worked out by the Commission on Standardization of Biological Stains in cooperation with the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Dept. of Agriculture. The dyes for which the methods are given in the present paper are: Fluorescein, eosin yellowish, ethyl eosin, eosin bluish, erythrosin, phloxine B, and rose bengal. For each of these dyes methods are given under the following headings: (1) identification or qualitative examination; (2) quantitative analysis; and (3) biological tests.**

The fluorane derivatives are readily reduced by titanous chloride and excellent checks are obtained in duplicate reductions, but due to the heterogeneous character of some of the commercial samples of the halogenated dyes of this group, large errors in the indicated dye content are encountered.[3] It has been found that the dye content of these samples may be determined more accurately and conveniently by the conversion of the dye into the form of the insoluble color acid.[4]

Certain members of this group are used as biological stains, but as there is considerable difference in the purpose for which the various members of the group are employed, different biological tests are necessary for each of the dyes listed. The biological tests employed at the present time are given for all these dyes, with the single exception of fluorescein.

The following methods, employed for the standardization of the fluoranes, include (1) the identification or qualitative examination;

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[3]See Holmes, W. C., Melin, C. G., and Peterson, A. R. Eosin B. Stain Techn., **7**, 121. 1932.

[4]See Holmes, W. C. The estimation of erythrosin. Color Trade Jour., **13**, 4. 1923

(2) the quantitative analysis; and (3) the biological tests for each individual dye.

## FLUORESCEIN, C. I. No. 766[5]

*Identification:* Fluorescein is the sodium salt of hydroxy-o-carboxyphenylfluorane. $C_{20}H_{10}O_5Na_2$. The absorption ratio of a solution of the dye (E at 500 m$\mu$/E at 530 m$\mu$) is approximately 36.5. E stands for the extinction coefficient; wave length is expressed in m$\mu$ and the solvent employed is one of equal volumes of distilled water and 95% alcohol to which has been added 5 drops of 1% sodium carbonate solution for every 50 cc. of solvent.

*Method of Analysis:* Dissolve an accurately weighed sample of about 0.4 g. of dye in 200 cc. of distilled water, add 10 g. of sodium tartrate, heat to boiling and titrate the hot solution with approximately N/10 titanous chloride solution in a current of carbon dioxide. During the titration the solution is kept well agitated by means of a mechanical stirrer. The disappearance of the yellow color indicates the end point. The dye content of the original sample is calculated as follows:

$$\frac{\text{cc. of N/10 } TiCl_3 \text{ used} \times 100}{53.164 \times \text{Wt. of Sample}} = \text{per cent of anhydrous dye.}$$

Note: 53.164 = cc. of N/10 titanous chloride solution necessary to reduce 1 g. of anhydrous fluorescein.[6]

*Biological Tests:* This dye has not been put on the certification basis and no biological tests have yet been developed for it.

## EOSIN YELLOWISH, C. I. No. 768

*Identification:* Eosin yellowish (eosin Y) is the sodium salt of tetrabromfluorescein, $C_{20}H_8O_5Br_4Na_2$. The absorption ratio of a solution of the dye (E at 505 m$\mu$/E at 535 m$\mu$) is approximately 0.92. The solvent used is the same as that described for fluorescein.

*Method of Analysis:* An accurately weighed sample of about 0.5 g. of the dye to be tested is dissolved in 400 to 500 cc. of distilled water. The solution is heated to the boiling temperature and the color acid precipitated with a little dilute hydrochloric acid (30 cc. N/10 acid). After cooling to room temperature, the color acid is filtered into a weighed Gooch crucible, washed with a very dilute solution of the same acid (approximately 0.2% strength), dried at 110° C., and weighed. From the weight of the precipitated color acid, the dye content of the original sample is calculated as follows:

$$\frac{\text{Wt. of Color Acid} \times 691.706 \times 100}{647.728 \times \text{Wt. of Original Sample}} = \text{per cent of anhydrous dye.}$$

Note: 691.706 = Molecular weight of eosin Y.
647.728 = Molecular weight of the color acid of eosin Y.

*Biological Tests:* Eosin Y is tested for two biological purposes: as a counterstain against hematoxylin and as a constituent of Wright's stain. In testing its properties as a counterstain it is made up in a solution of 0.1% to 0.5% strength in 25% alcohol

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[5]This abbreviation stands for the number in the "Colour Index", published by the Society of Dyers and Colourists, Bradford, Yorkshire. 1924.

[6]The theoretical number of cc. of N/10 titanous chloride solution required for the reduction of 1 g. of any dye may be calculated by means of the following formula:

$$\frac{\text{No. of H equivalents required for the reduction of 1 mol of dye} \times 10 \times 1000}{\text{Molecular Weight of the Dye}}$$

(see page 124, Biological Stains, 2nd ed.[7]). It is tested on paraffin sections of tissue fixed in Zenker's solution.

In testing its behavior as a constituent of Wright's stain the formula given on page 147 of Biological Stains is employed and the compound stain, after dissolving in methyl alcohol, is applied to blood smears for one to three minutes without diluting and is then diluted on the slide in distilled water with two to six minutes application of the diluted stain. It is washed and dried and examined under the microscope. In judging a good Wright's stain, special attention is given to the question of whether the red cells show a desired yellow pink or whether all the granules of the various leucocytes are differentially stained.

## ETHYL EOSIN, C. I. No. 770

*Identification:* Ethyl eosin is the potassium (or sodium) salt of the ethyl ester of tetrabromfluroescein, $C_{22}H_{11}O_5Br_4K$. The absorption ratio of a solution of the dye (E at 515 m$\mu$/E at 545 m$\mu$) is approximately 0.86. The solvent employed is the same as that described for fluorescein.

*Method of Analysis:* The titration of this dye with titanous chloride is not satisfactory and the color acid cannot be precipitated directly. Assuming that ethyl eosin is the only dye present, the following method may be used:[8] An accurately weighed sample of about 0.5 g. of the dye to be analyzed is boiled 2 or 3 hours with 500 cc. of distilled water containing 18 cc. of normal sodium hydroxide in order to convert the ethyl ester of eosin into the sodium salt. The solution is cooled, filtered on a small filter, and washed thoroly with cold water. The combined filtrate and washings are acidified with 4 cc. of 6 N hydrochloric acid and allowed to stand 30 to 60 minutes. The precipitated color acid is filtered on a weighed Gooch crucible, washed with an approximately 0.2% solution of HCl, dried at 110° C., and weighed. The dye content of the original sample is calculated as follows:

$$\frac{\text{Wt. of Color Acid} \times 713.848 \times 100}{647.728 \times \text{Wt. of Original Sample}} = \text{per cent anhydrous dye.}$$

Note: 713.848 = Molecular weight of ethyl eosin.

647.728 = Molecular weight of the color acid of eosin Y.

*Biological Tests:* Ethyl eosin is tested for the demonstration of Negri bodies in the central nervous system of rabid animals. Pieces of such tissue, fixed in Zenker's solution, are embedded in paraffin and sectioned. They are then stained either by the Harris or by the Stovall technic as given on page 126 of the 2nd edition of Biological Stains. By this technic, the nerve cells should stain blue, the Negri bodies red.

## EOSIN BLUISH, C. I. No. 771

*Identification:* Eosin bluish (eosin B) is the sodium salt of dibromodinitrofluorescein, $C_{20}H_6N_2O_9Br_2Na_2$. Samples of this dye have been of such divergent character that no conclusion as to a reliable absorption ratio has been made. It was found in a recent investigation by the Color and Farm Waste Division[9] that samples of this stain are

[7]Conn, H. J. Biological Stains. *2nd Ed.* Biological Stain Commission, Geneva, N. Y. 1929.

[8]This "assumption" is emphasized, as so many commercial samples of this dye have been found to contain appreciable proportions of eosin Y. Suitable methods for the evaluation of eosin and ethyl eosin in samples of the latter dye are given in a paper "The evaluation of ethyl eosin" by W. C. Holmes and J. T. Scanlan, Stain Techn., 2, 101. 1927.

[9]See footnote 3.

neither pure 4, 5-dibromo-2, 7-dinitrofluorescein, nor 2,7-dibromo-4,5-dinitrofluorescein. They may consist principally of mixtures of the two isomeric dibromodinitrofluoresceins or of mixtures of various other bromonitro derivatives.

Absorption alone is insufficient for the characterization of samples of eosin B. It is necessary to determine bromine and nitrogen values to obtain a more definite clue to their character.

*Method of Analysis:* Dissolve an accurately weighed sample of about 0.5 g. of the dye to be examined in approximately 500 cc. of water, heat to boiling and add slowly with constant stirring, 2 or 3 cc. of 6 N hydrochloric acid. Cool the hot solution to room temperature and allow to stand at least 1 or 2 hours. Filter the precipitated color acid on a weighed Gooch crucible, thoroly wash with a 0.2% solution of hydrochloric acid, dry at 110° C., and weigh. Calculate the dye content of the original sample from the weight of the color acid by means of the following formula:

$$\frac{\text{Wt. of Color Acid} \times 623.890 \times 100}{579.912 \times \text{Wt. of Original Sample}} = \text{per cent anhydrous dye.}$$

Note: 623.890 = Molecular weight of eosin B.
579.912 = Molecular weight of the color acid of eosin B.

*Biological Tests:* Eosin B is tested as a counterstain for hematoxylin by the following technic: Material fixed in either Bouin's fluid or Zenker-formol is embedded in paraffin and cut 10 $\mu$ in thickness. Sections are mounted on slides with Mayer's albumen fixative. They are then run down to water, stained for from 5 to 10 minutes in Mayer's hemalum, dipped once or twice in tap water, then holding them in forceps dipped into the solution of eosin once, twice or three times, dehydrated and mounted.

The solution of eosin is 0.5% solution of the stain in 20% alcohol. If it overstains, dilute with distilled water. In going quickly from Mayer's hemalum into the eosin a precipitate is formed in the latter and the color is also precipitated into the tissue so that it does not wash out readily.

## ERYTHROSIN, C. I. No. 773

*Identification:* Erythrosin is the sodium salt of tetraiodofluorescein, $C_{20}H_6O_5I_4Na_2$. The absorption ratio of a solution of the dye (E at 515 m$\mu$/E at 545 m$\mu$) is approximately 1.04. The solvent used is the same as that described for fluorescein.

*Method of Analysis:* Precipitate the color acid of the dye according to the method given for eosin B. From the weight of the color acid, the dye content of the original sample is calculated as follows:

$$\frac{\text{Wt. of Color Acid} \times 879.770 \times 100}{835.792 \times \text{Wt. of Original Sample}} = \text{per cent of anhydrous dye.}$$

Note: 879.770 = Molecular weight of erythrosin.
835.792 = Molecular weight of the color acid of erythrosin.

*Biological Tests:* Erythrosin is tested in the Jackson stain for plant anatomy given in STAIN TECHNOLOGY, 1, 33. In employing this procedure as a test for erythrosin, plant buds or other botanical material containing both lignified and non-lignified cell walls are fixed in one of the usual fixatives, embedded in paraffin and sectioned. After removing the paraffin they are stained 15 minutes in 1% crystal violet in distilled water. Then, after rinsing and dehydrating, they are differentiated one to five minutes in a saturated solution of erythrosin in clove oil. The sections are then cleared and mounted. By this method the non-lignified tissues should stain with erythrosin and the lignified walls with crystal violet.

## PHLOXINE B, C. I. No. 778

*Identification:* Phloxine B is the sodium salt of tetrabromtetrachlorfluorescein, $C_{20}H_2O_5Cl_4Br_4Na_2$. The absorption ratio of a solution of the dye (E at 515 m$\mu$/E at 545 m$\mu$) is 0.97. The solvent employed is the same as that described for fluorescein.

*Method of Analysis:* Precipitate the color acid of a weighed sample of the dye to be tested by the method outlined for the analysis of eosin B. From the weight of the color acid, the dye content of the original sample is calculated by means of the following formula:

$$\frac{\text{Wt. of Color Acid} \times 829.502 \times 100}{\text{Wt. of the Original Sample} \times 785.524} = \text{per cent of anhydrous dye.}$$

Note: 829.502 = Molecular weight of phloxine B.

785.524 = Molecular weight of the color acid of phloxine B.

*Biological Tests:* Phloxine B is tested by the Mallory phloxine and methylene blue technic, by the procedure given on pages 131–2 of Biological Stains, 2nd edition. A satisfactory stain is judged by the pink color of the cytoplasm, while the nuclei are colored by the methylene blue.

## ROSE BENGAL, C. I. No. 778

*Identification:* Rose bengal is the sodium salt of tetraiodotetrachlorfluorescein, $C_{20}H_2O_5I_4Cl_4Na_2$. The absorption ratio of a solution of the dye (E at 540 m$\mu$/E at 570 m$\mu$) is 0.92. The solvent employed is the same as that described for the identification of fluorescein.

*Method of Analysis:* The method for the determination of rose bengal is identical with that of eosin B, except that in the calculation of the dye content of the original sample, the following formula is to be used in place of the one given for the latter dye.

$$\frac{\text{Wt. of Color Acid} \times 1017.566 \times 100}{\text{Wt. of Original Sample} \times 973.588} = \text{per cent of anhydrous dye.}$$

Note: 1017.566 = Molecular weight of rose bengal.

973.588 = Molecular weight of the color acid of rose bengal.

*Biological Tests:* Rose bengal is tested in Conn's technic for staining bacteria in soil. The procedure followed is the one given on page 133 of Biological Stains, 2nd edition, except that if unsatisfactory results are obtained with the amount of calcium chloride (0.01%) recommended in the formula, variations in the amount of this salt are tried in order to secure better results on the theory that increasing the amount of calcium in the solution intensifies the action of the stain. A sample is judged satisfactory which permits a deep staining of the bacteria without sufficient coloring of the soil or dead organic matter to obscure the micro-organisms. In making the test, soil known to have an unusually large number of bacteria is employed.

# THE MASSON TRICHROME STAINING METHODS IN ROUTINE LABORATORY USE

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ABSTRACT.—**A resumé of Masson's trichrome staining methods is given, with detailed directions for carrying out all of his procedures. The results obtained thru their use in a routine laboratory are discussed at length, as well as the fact that they also work very well on tissues fixed in ways other than those he prescribes, and stained with chemicals and dyes other than those he uses. The fact is stressed, however, that the closer one adheres to his precepts, the better will be the results.**

**The stains described include his hematoxylin-phloxine-saffron, his iron-hematoxylin-ponceau- anilin-blue, his variants of this stain (of which the light green stain is excellent), his metanil yellow and his modification of the familiar Van Gieson technic. All these stains are based on familiar laboratory methods, improved and rendered trichrome, so that they present no great obstacles in technic.**

**Of the methods cited, the writer prefers the "light green" procedure. Sections are prestained in Regaud's iron-hematoxylin, followed by a mixture of ponceau de xylidine and acid fuchsin. This is followed by mordanting in phosphomolybdic acid and the sections are finally stained in light green. The results are very precise and pleasing and afford immediate orientation as the connective tissue is green, the nuclei black or dark purple, the cytoplasm of the cells is in varying tones of red. The method may be used after fixation in almost any good medium; altho the results are not as brilliant as those obtained after one of Masson's prescribed fixations, it is believed that they are even then superior to those following the routine hematoxylin-eosin method.**

## INTRODUCTION

Masson presented a régime of trichrome staining at the Chicago meeting of the International Association of Medical Museums in 1929, the paper being translated and published in the *Journal of Technical Methods and Bulletin* of that society[1]. Prior to this, Masson's technic had appeared in French publications and, altho familiar to the French laboratory workers, it was as yet practically unknown in America.

[1]Masson, P. Some histological methods. Trichrome stainings and their preliminary technique. J. Tech. Methods and Bul. Int. Assn. Med. Mus., **12**, 75. 1929.

A number of Americans have now become aware of its excellence and have been using it with success, but their number is still far too small, and the superiority of these staining procedures over older and more familiar systems has not been fully realized. It is the purpose of this paper to discuss the technic as it works out in a routine surgical pathology laboratory and to dispel, if possible, the haze of diffidence that seems to obstruct its adoption at the present time. It is popularly supposed to be very formidable, to take a great deal of care, time, and labor, but this is not the case. While it is true that it requires a bit more attention than the simpler dichrome methods in vogue, this is amply made up for by the results obtained, which are far superior to those of the older procedures.

Masson is a technician of very high ideals; therefore his description of his methods includes special fixatives, special methods of embedding, a new way to fix paraffin sections to glass slides and minute details for the preparing of stains. He wished to allow no opportunity for failure and, therefore, gave his entire regimen in this paper, from the fixation of the tissue to the final mounting of the sections on the slides. It is not generally known that, while his methods do give the best results, more usual procedures will also afford preparations that are vastly better than the usual laboratory products. For this reason it is proposed to discuss the outcome of several months of practical experience in this laboratory from the standpoint of routine procedure and mass production. The reader is cautioned in advance that he need not expect to rival Masson unless he follow the prescriptions of his paper in every detail, but is is also certain that if he follow the lines to be laid down in this resumé he will not be disappointed.

## Preliminary Treatment

*Fixation:* Masson recommends a Bouin's solution composed of commercial formalin (40% formaldehyde)—10 parts, 2% aqueous solution of trichloracetic acid—2 parts, and a saturated aqueous solution of picric acid—30 parts. The solution is made up with water and the picric acid is then added to saturation, which is complete at room temperature in three days; the excess may be then filtered out, or left as a sediment in the bottle. He recommends three days' fixation, but one day will suffice in routine practice if the blocks be not over 5 mm. thick. The fixative is not washed out of the tissue, the pieces being transferred directly to 80% alcohol.

Ordinary formalin is not supposed to give good results unless it be reinforced with potassium bichromate. Masson recommends a 3% bichromate solution in water—90 parts, to which 10 parts of strong

*neutral* formalin is added just before using. We have found that 10% neutral formalin alone, 10% formalin in 95% alcohol, or fixation in the usual Zenker's fluid will all give excellent results, even if the color scheme is not the standard that is obtained after Masson's prescribed fixations. Formalin-alcohol tends to shrink the tissue somewhat, but the results are perfectly compatible with laboratory routine diagnosis. If bichromate has been used, it should be thoroly washed out of the tissues by rinsing for 24 hours in running water and, in the case of Zenker fixation, the mercury salts removed from the sections later, with weak alcoholic iodine followed by weak aqueous sodium thiosulphate.

We are using two methods of fixation—a "rapid" and a slow one. In the former case thin blocks of tissue are placed in alcohol-formalin for five or more hours, transferred to 95% alcohol over night, to absolute alcohol during the following morning, to chloroform during that afternoon, to chloroform saturated with paraffin over night and embedded in paraffin the following morning by immersion for five hours in two changes of melted paraffin, followed by blocking in paper forms. In the slow method 10% neutral formalin, formalin-bichromate, Bouin's, or Zenker's fluids are used, often blocks are run in all of these for comparison, as fixation makes marked differences in some tissues. We fix in these for 10 or more hours and follow by a day of washing if bichromate has been used. The tissues are transferred to 80% alcohol over night, to 95% alcohol during the next day, to absolute alcohol during the ensuing night, to chloroform the next day, to chloroform-paraffin the following night and melted paraffin the next morning, when the blocks are embedded after five hours' impregnation. The "rapid" method is not very fast at that, depending largely upon the working day of the technician in the laboratory. It can be somewhat shortened in the case of very small blocks by curtailing the time they remain in the various agents. We do not use acetone on account of shrinkage, which is much more marked with small blocks in that case. An embedding machine, or tissue-changer, which mechanically transfers the blocks from one dehydrating agent to the next at any specified time, will further shorten the process, as it is independent of train schedules, lunch hours, or the time of day or night.

*Embedding:* Masson's method of embedding requires more time than is usually allowed by the busy and inquiring surgeon and compatible with a rapid diagnostic service, but for research and teaching purposes it is undoubtedly excellent and insures even impregnation and dehydration. The reader is referred to Masson's original article, if he wish to employ this more leisurely procedure.

We try to make the actual embedding in paraffin as rapid as possible, to avoid exposing the tissue to heat and hardening. Blocks are transferred from chloroform-paraffin, as indicated above, and placed in melted paraffin at 56° to 58°C. for five hours, two changes of paraffin being used. Masson uses a paraffin of a much lower melting point, but we are constrained by circumstances to use this harder material.

*Sectioning:* It is essential to cut thin and even sections at about 5 microns with a knife that is irreproachably sharp, as Masson's stains are intense and will overstain thick or ridgy sections.

*Fixing the Sections on the Slides:* Masson has a special method for fixing the sections to the slides. He uses a mixture of 0.05 g. of gelatin, soaked in 2 to 3 cc. of distilled water, which is then boiled and diluted up to 25 cc. with cold distilled water, and filtered thru filter paper. Sections are cut out of the dry ribbon with a scalpel, the edge of this is moistened in the solution and the section picked up by one edge and laid, with its shiny side down, on the meniscus of a few drops of the gelatin solution on a slide. The slide is then placed on a warm plate at about 45°C. until the section flattens out, which should not take more than 10 or 15 seconds, as the heating might otherwise cause distortions in the connective tissue. The slide is then placed in a drying oven of approximately 45° C. for several hours; if the staining method to be used later depends upon hot solutions it is best to expose the drying sections to formalin vapor from an open jar of 10% formalin stood in the oven. This hardens the gelatin, as in the case of photographic plates in warm weather.

We find that the ordinary egg-albumin-glycerol adhesive may be smeared on slides and the sections floated onto these from warm water, in the classic fashion, without disturbing the results. We also find that Masson's solution may be made up ten times stronger and used in the same manner as the egg-albumin-glycerol. If one method is unsatisfactory, another will succeed; we find, however, that they all work equally well.

*Celloidinization:* Sections fixed in Bouin's solution and intended for staining with saffron should be coated with a pellicle of celloidin in order to filter the saffron and increase its precision, while at the same time reducing its intensity. Dry celloidin is dissolved in the proportion of 0.3 g. to 100 cc of a mixture of equal parts of ether and absolute alcohol. The slides are dipped into this, their backs wiped dry with a clean cloth. They are then submerged in 80% alcohol, which hardens the pellicle over the sections, then stood in running water until wanted. We find that this is necessary only in the case of Bouin-fixed tissue; other fixations render the saffron stain paler and do not require the pellicle.

## Staining Methods

### 1. saffron method

This is essentially an hematoxylin-eosin technic to which saffron staining is added to give the third color.

*Nuclear Stain:* This consists of a preliminary stain with hemalum made up by boiling a saturated solution of potassium alum (Baker, Merck) in distilled water and adding 0.2 g. of powdered hematein (Geigy, Krall[2]). It is then cooled, filtered and 2% of glacial acetic acid added. It is good for about one month, after which it loses strength thru precipitation. As we prefer Harris' hematoxylin we have substituted it for Masson's hemalum and it works very well.

*Cytoplasmic Stain:* A 1% aqueous solution of phloxine or erythrosin serves this purpose; we have been using the former and have also found that ordinary water-soluble yellowish eosin will work well. As saffron is yellow, better contrasts are obtained thru the rosier eosins, or relatives of eosin. The slides should be stained in this solution for five minutes or less. In using 5% aqueous yellowish eosin, according to Mallory's technic, we merely pour it on and off the sections.

*Connective Tissue Stain:* Here an infusion of the pistils of the *Crocus sativus* (Gâtinais saffron, Krall) is employed. These must be of the current year's crop, limber and moist, not dry and brittle. (They may be obtained from Eimer & Amend in New York.) Spanish saffron, as used by our foreign element for coloring cakes, is usable but not as good. Two grams of the pistils are heated for an hour on the steam bath in 100 cc. of tap water (we have found distilled water rather better), a deep orange infusion resulting. This is then cooled and 1 cc. of strong formalin and 1 cc. of 5% aqueous tannic acid solution are added. The infusion should be bottled and kept in a dark, cool place, where it will retain its strength for three weeks if not in use, or about one week if being used in a staining box and poured back into the bottle.

*Technic:* The celloidin-coated sections should be stained for 5 to 10 minutes in hemalum or hematoxylin, rinsed in water and differentiated in a solution of 5 drops of hydrochloric acid to 100 cc. of 95% alcohol until the tissue markings are visible. We prefer to use a 1% solution of the acid in water, which obviates changing the slides from water to alcohol and keeps the solutions isotonic. The sections are then blued in running water. The cytoplasm is next stained by immersion in the phloxine, erythrosin or eosin solution for 5 minutes; if the solution be diluted five volumes and the staining

[2]The b ands of dyes mentioned here and below are those specified by Masson. In the case of common stains, however, other brands have been found equally satisfactory.

time increased to 30 minutes the results will be more precise and delicate. The slides are then rinsed and drained. The connective tissue is stained with the saffron for from 2 minutes in fresh saffron infusion to 5 minutes or more if it be old. This infusion may be dropped onto the sections from a medicine dropper and then discarded, or the slides may be stained in a glass box and the infusion saved for another batch. This is simpler, but the infusion deteriorates more rapidly. Following this stain, the sections should be rapidly dehydrated by flooding them one by one, from a rapidly-flowing dropping-bottle or flask, with *absolute* alcohol (weaker percentages *extract the saffron*). If the staining-box method be used, several changes of absolute alcohol must be employed. The celloidin is dissolved by this step and the sections are ready to be run into xylol or toluol and mounted in Canada balsam. If the fixation has been other than Bouin's fluid, the celloidinization may be omitted and the staining time somewhat increased in the saffron infusion. Water is the enemy of good saffron stains, so that rapid dehydration is the secret of success with this method.

### 2. ANILIN BLUE METHOD

This is an improved Mallory technic and the results obtained after fixing the tissue in Zenker's fluid, Bouin's fixative, or Masson's bichromate-formalin solution are very beautiful and should be considered as constituting the standard for the stain. Tissue fixed in formalin-alcohol or neutral formalin, however, also gives very lovely results, altho the color scheme departs somewhat from Masson's standard, inasmuch as the acid fuchsin and ponceau do not color the cytoplasm as intensely and are found chiefly in the erythrocytes, which are bright vermillion; otherwise, the cytoplasm of the cells may be faintly pink, or pinkish-gray. At all events, we consider the results on such tissue distinctly superior to those obtained with the older dichrome routines.

*Nuclear Stain:* This is the standard for all the following methods. A Regaud's iron-hematoxylin stain is carried out as follows. One gram of hematoxylin is dissolved in 80 cc. of hot distilled water, the solution is cooled and 10 cc. of 95% alcohol and 10 cc. of glycerol are added. Sections are first mordanted in a 5% aqueous solution of iron alum in the drying oven at 45 to 50° C. for 5 minutes. They are then washed at the tap and stained for 5 minutes in the hematoxylin solution at 45 to 50° C. It is here that the Masson gelatin solution is useful if the sections show a tendency to become detached from the slides in the warm fluids. They are now evenly black and overstained and should be differentiated in a saturated alcoholic solution

of picric acid (95% alcohol)—2 parts, to one part of 95% alcohol until the nuclei alone retain the stain and the collagen fibers are no longer gray, which may be ascertained with the microscope. They are then washed for 15 minutes in running water. We have found that unless one wishes to have "supersections", it is sufficient to differentiate long enough to render the tissue details clearly visible. This takes about 5 minutes for ordinary tissue; thyroid, bone and skin sections may take as much as 15 to 20 minutes for good differentiation. The wash in running water may be shortened slightly at the expense of nuclear precision, if one be in a hurry. Masson's point in extracting all the color from the collagen is to insure pure blues and greens, or yellows, and to avoid muddy effects; a light gray residue in this tissue, however, blends so well with blue or green that one need not be too particular, altho in the case of metanil yellow the color is definitely interfered with if the differentiation is incomplete.

*Cytoplasmic Stain:*

(A) Ponceau de xylidine: A 1% solution of this dye (Krall; Eimer & Amend) is made up in distilled water to which 1% of glacial acetic acid has been added. Sections stained in iron-hematoxylin and washed in water are brought into this for 5 minutes, rinsed with distilled water and carried into the connective-tissue stain.

(B) Ponceau-Acid Fuchsin: Two parts of the above ponceau solution are mixed with one part of a 1% acid fuchsin solution in 1% of acetic acid and the sections stained in this mixture for 5 minutes. This gives a wider range of reds, varying from vermillion to ruby or rose-red, according to the cytoplasm stained. It is, therefore, prettier and more valuable, bringing out subtle differences between the various cells. It is advantageous to dilute either A or B with 10 volumes of distilled water with 1% acetic acid added and to stain for an hour or more in this weaker solution, which keeps well and may be used repeatedly. This gives even more delicacy and precision. It is to be noted that acetic acid is used in all of these cytoplasmic stains to prevent the extraction of color by plain water.

*Connective-tissue Stain:* Sections dyed with hematoxylin and one of the above cytoplasmic stains are mordanted in 1% aqueous phosphomolybdic acid for 5 minutes, rinsed in distilled water and stained for 5 minutes in an anilin blue solution made by boiling 2 g. of anilin blue in 100 cc. of distilled water, adding 2 cc. of glacial acetic acid, cooling and filtering. The sections are rinsed in distilled water and again set in the phosphomolybdic acid bath for 5 minutes in order to extract any excess blue. They are then rinsed rapidly and placed in 1% acetic acid for 2 minutes, after which they are run

briefly thru tap water and alcohols of ascending percentages into xylol or toluol, to be mounted in balsam.

Results: Nuclei black, or deep violet, cytoplasm varying shades of red, connective tissue and mucus blue.

### 3. LIGHT GREEN STAIN

This is precisely similar to the preceding method excepting that light green replaces anilin blue in like amount (Krall; Eimer & Amend). One should omit the second immersion in phosphomolybdic acid as the green is not as intense as the blue and needs no differentiating. The blue is replaced in this instance by a pleasant grass green, which is more pleasing, precise and restful to the eye. We are using this method, combined with the fast ponceau-acid fuchsin, as our laboratory routine stain.

### 4. ACID-FUCHSIN-METANIL-YELLOW

This stain is intended for immediate use, not for permanent collections, as it tends to fade. It is not used much in our laboratory as the results obtained with the other methods are very satisfactory and more permanent. It is merely cited here for the sake of completeness.

*Cytoplasmic Stain:* Sections from material fixed in *Bouin's fluid* are stained with Regaud's iron-hematoxylin and differentiated in picric acid alcohol, they are then washed and counterstained with a 1% aqueous solution of acid fuchsin with 1% acetic acid, for 5 minutes and differentiated with the phosphomolybdic acid solution.

*Connective-tissue Stain:* The slides are next transferred to a saturated solution of metanil yellow in distilled water, *without washing*, for 30 seconds to 2 minutes. They are then rinsed in distilled water, dehydrated and mounted.

Results: Nuclei blackish, cytoplasm rose-red, connective tissue yellow.

### 5. MODIFIED VAN GIESON STAIN

*Cytoplasmic Stain:* Sections stained with Regaud's iron-hematoxylin are washed and differentiated for a short time in picric acid alcohol, or aqueous picric acid. The differentiation should not be complete, as the picric acid in the connective-tissue stain that follows will continue the extraction of hematoxylin. This is a matter of practice and one can soon learn the proper point to discontinue the differentiation. They are then placed for 5 minutes in a mixture of 2 cc. saturated aqueous metanil yellow (distilled water) to 98 cc. of 1% acetic acid.

*Connective-tissue Stain:* The sections are mordanted in 3% potas-

sium bichromate solution for 5 minutes and, without rinsing, transferred directly to a 1% solution of acid fuchsin in saturated aqueous picric acid for 30 to 60 seconds, after which they are rinsed very briefly in distilled water. They are next placed in 1% acetic acid for 5 minutes, after which they are finished and mounted in the usual manner. The acetic acid bath removes the picric acid without harming the fuchsin or metanil yellow.

Results: Nuclei blackish, cytoplasm yellow (its color varying with the nature of the cells and the length of time they were stained), and collagen intense carmin red. The results are similar, but superior to those of the ordinary Van Gieson stain and the preparations are more permanent. We find this stain to be very precise and brilliant and applicable to a variety of fixations, it is to be recommended in all instances where a Van Gieson method is indicated.

*Note:* It will be noted, in passing, that Masson has made a *tour de force* in these last two stains. Using essentially the same dyes, he completely reverses the staining affinity of these by appropriate treatment; in the one the metanil yellow stains the connective tissue, in the other it stains the cytoplasm, while the acid fuchsin is also reversed.

## Discussion

Of the methods above described, we have found the light green combination (No. 3) the most valuable and are using it as a routine method for everything. One is immediately oriented by the bright green connective tissue, the topography being beautifully picked out. The variations in the cytoplasmic stain, occasioned by the mixture of ponceau and acid fuchsin, are also valuable in distinguishing between cells of different types, some being vermillion, some rose-red, some grayish-pink. A better stain for the intercellular bridges of the stratum spinosum in the epidermis could hardly be imagined; these stand out bright red, with definite red nodules at their mid-points, while the cells are paler. Immature colloid in the thyroid usually stains green, while mature colloid is colored red, a valuable point in connection with hyper- or hypoplastic conditions and the diagnosis of Graves' disease. Nerve trunks are stained in three colors: nuclei blackish, connective tissue sheaths in green, neuraxones and sheaths of Schwann in red. Mucus stains green in the goblet cells of the intestinal mucosa, the cells themselves being pink or red. One might cite such advantages indefinitely.

If the sections are not unduly exposed to light and are handled in the ordinary routine fashion, to be filed in reasonably light-tight boxes or cabinets, there is no appreciable fading of the stains and if

there were, most of them are so intense that a little stepping-down will do no appreciable harm.

Visitors to this laboratory have been so enthusiastic about our results with these methods that there is little doubt that the reader will share their enthusiasm. At first, a microscopist may be a little dismayed by the novel color scheme and the reversal of the familiar colors; bright green connective tissue is a distinct novelty to the "red-and-blue" man, who is accustomed to hematoxylin-eosin, or eosin-methylene blue. The step from blue to green is not so unsettling, however, so that those who are accustomed to the color scheme of Mallory's connective tissue stain find its counterpart in Masson's anilin-blue method and easily make the transition to his light green method.

All the technics here quoted have been carefully worked out by Masson over a long period of time and all are, therefore, good and reliable. The reader may rest assured that if he follows Masson's directions conscientiously he will, with a little practice, succeed in getting excellent results. It should also be noted that if one be hampered by one thing or another and cannot get the prescribed stains or fixatives, one need not follow these conditions too slavishly, for the procedures will work well on tissue fixed in the usual way and, even if the results differ slightly from the standard, they will be superior to those obtained with the older dichrome methods. One may substitute one's favorite hematoxylin or iron-hematoxylin for those used by Masson and still get fine preparations; one may use other eosins than the ones he prescribes; it is only in the case of the saffron that one must be very particular as to the brand used, for the fresh Gâtinais product is practically a *sine qua non*. Masson specifies distilled water in many instances, but if the city water supply be good (pure and unchlorinated) it may work as well—one need but try it out. We find the New York City supply adapted to many of our needs, without recourse to distilled water.

The time element in these methods need not deter nor intimidate the technician, one soon becomes accustomed to the schedules and to having the sections standing about the room in the various baths and stains and one finds that, in the end, the laboratory schedule runs perfectly smoothly once one is accustomed to the change. The advantages that accrue from the use of the methods more than offset the extra time consumed in carrying them out.

The author is much indebted to Miss Anna Mary McDowell for her invaluable aid in experimenting with these methods in our laboratory.

# THE TINCTORIAL BEHAVIOR OF THE CAPSULE OF CLOSTRIDIUM TETANI DURING SPORULATION[1]

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ABSTRACT.—**Bacterial smears are stained by a technic previously described by the author. In this technic Wright's stain is used and is allowed to evaporate almost to dryness without diluting; it is then washed off rapidly with a buffer solution (pH 6.5) and is quickly dried by means of fanning. Using this technic, various structures are observed in the sporulation of *Clostridium tetani* and *C. Welchii* which do not seem to have been described before. Descriptions of these structures are given in the present paper.**

*Clostridium tetani* (Nicolaier) Holland[2] has been stained by the method recently described by Churchman and Emelianoff[3], the principle feature of which is staining with Wright stain, nearly drying without dilution and rapid washing with buffer solution at pH 6.5. By this method a definite capsule is demonstrable. The soma stains deep blue or bluish-black, the capsular membrane dark pink. The capsular substance is, as a rule, very lightly stained, sometimes pinkish, sometimes light purple. Often, however, it is almost clear. It is noteworthy that the vegetative forms of this organism vary greatly in size; but whether they are long or short the proportion of soma to capsule remains roughly the same. The picture usually obtained is shown in the upper three individuals in Fig. 1. Often the bacterial body, or soma, lies not in the middle of the capsule but well over to one side; (see lower right-hand organism Fig. 2). Another variation from the typical picture is furnished by occasional individuals like those shown in the lower row in Fig. 2 in which the capsular membrane lies practically upon the soma. This exceptional picture may be due either to shrinkage of the capsule or to swelling of the soma, both of which phenomena appear to be clear in the illustration. The picture presented by the vegetative form of *Clostridium tetani* when stained by our technic is thus very similar to that shown by one of the best known of all capsulated organisms, *Clostridium Welchii* (*Bacillus aerogenes capsulatus* the cause of gas gangrene) when

[1]Work aided by a grant from the Josiah Macy, Jr. Foundation.

[2]Six strains were studied from the laboratory of Dr. Elser.

[3]Churchman, J. W. and Emelianoff, N. V. A new method of staining bacterial capsules. Proc. Soc. Exp. Biol. & Med., **29**, 514–15. 1932. A study of the bacteria. capsule by new methods. J. Exp. Med. **57**, 1933. See Abstract in Stain Techn. **8**, p. 75.

## Plate I

Drawn at magnification of about 6,000 ×. Background (usually purplish-pink, sometimes with purplish-black granules of precipitated stain) omitted.

Fig. 1. Above: Three vegetative forms of *Clostridium tetani;* capsular membrane pink; soma blue; capsular substance clear or nearly clear.

Below: Individual in sporular form stained by Burke's modification of Gram's method; vegetative form stained by methylene blue; no capsule visible.

Fig. 2. Varieties of staining encountered. Above; left-hand organism, slightly eccentric position of soma; right-hand organism, granular change of soma.

Below: At left, soma swollen and capsular membrane lying upon it (this type is seen only rarely). Middle organism, organism shrunken, capsular substance obliterated, capsular membrane lying practically upon the slender soma. At right, soma lying well to the side of the capsule (a picture frequently seen).

Fig. 3. Various types of fission. Right-hand organism in upper row: purplish-black granules of precipitated stain lie on the capsular membrane. Left-hand organism, lower row, shows an unusual type of budding rarely encountered.

Fig. 4. Early stages in sporulation. At first (upper left-hand organism), a change in morphology only, tinctorial reactions as in the vegetative form. Then, thickening of capsule which now stains almost black. This change is usually seen only about the spore, but occasionally (lower organism) is seen also about the rest of the soma.

Fig. 5. Later changes; horseshoe developing about spore: rest of soma receding.

Fig. 6. Still later changes: Further recession of rest of organism as spore develops.

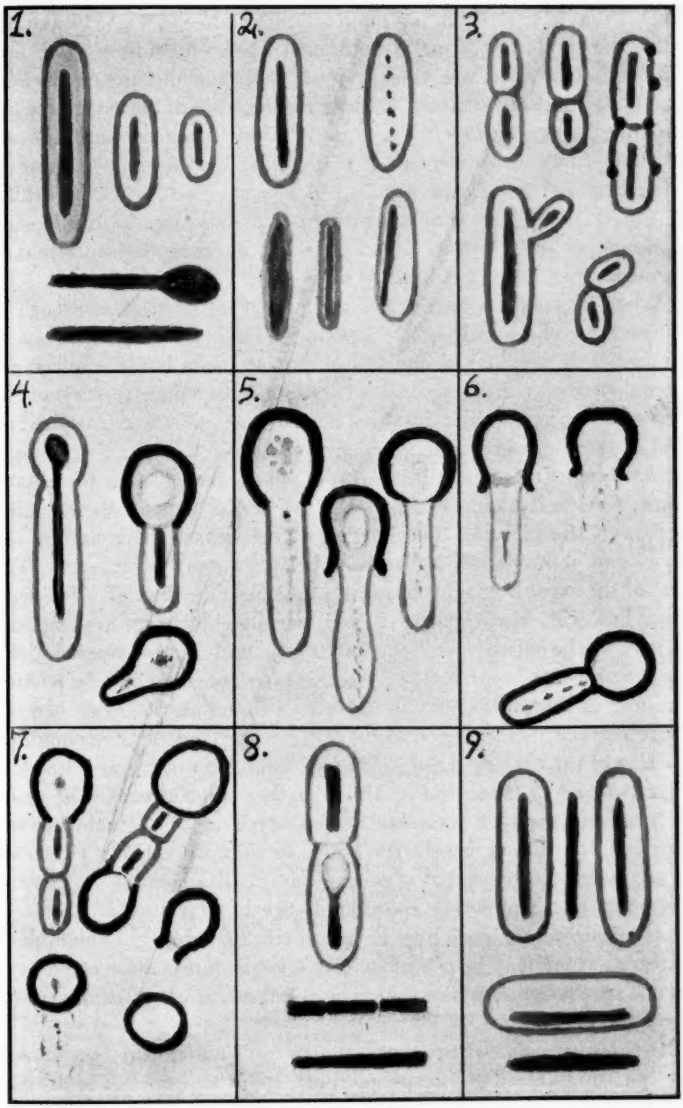
Fig. 7. Two organisms are seen in fission, one with a spore developing from either segment. The last stages of sporulation are also seen. The black stained horseshoe, containing the spore, may be seen without any evidence of the rest of the organism. Or the horseshoe may be closed into a circle, sometimes with the ghost of the rest of the organism just visible, as in the individual in the lower left-hand corner.

Fig. 8. Above: *Bacillus anthracis* stained by our method for comparison with *Clostridium tetani.* Lower segment sporulating; upper segment still vegetative.

Below: individuals stained by Burke's modification of Gram's method and by methylene blue; no capsule to be seen.

Fig. 9. *Clostridium Welchii* for comparison with *Cl. tetani.* Above: two individuals stained by our method. Between them an individual stained by methylene blue. Below: another individual stained to show the capsule; eccentrically placed soma. At bottom of figure, individual stained by Burke's modification of Gram's stain; no capsule to be seen.

Notice that in the vegetative form this well-established capsulated organism has (when stained by our method) a structure quite similar to that of the supposedly non-capsulated *Cl. tetani.* (See also Figs. 10 and 11).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similarly stained. This similarity is evident from examination of Figs. 1 and 9, and of the photomicrograph shown in Figs. 10 and 11. Also, the similarity of picture obtained when *Clostridium tetani* and *Clostridium Welchii* are thus stained, with the picture of *Bacillus anthracis* similarly stained, is clear from an examination of Fig. 8.

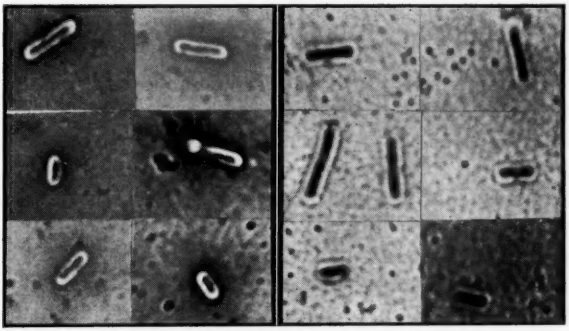
While fission of *Clostridium tetani* is taking place the participation of the capsule in the process is made clear by the pink staining of the partition between the dividing segments. This fact is illustrated in Fig. 3. Attention should be called to an atypical form of fission occasionally encountered in which what appears to be a bud is formed. (See lower left-hand organism Fig. 3).

When sporulation begins and as it proceeds a striking change in the reaction of the capsule to the stain used in our technic takes place. At first only swelling at one end of the organism is to be observed, accompanied by a slight constriction somewhat below the end of the organism. This swelling involves the capsule and the corresponding end of the soma. But the staining reactions of the organism remain undisturbed; the soma still stains blue-black, the capsular membrane pink. (See left-hand organism Fig. 4). Soon a notable alteration occurs in the picture. The portion of the capsule surrounding the spore now stains purplish-black and becomes greatly thickened. The rest of the capsule usually continues to stain pink (Fig. 4). Occasionally, however, the thickening and purplish-black staining appear to involve the entire capsular membrane. (See lower organism Fig. 4). As the process of sporulation advances the thickening and deep black staining of the portion of the capsule surrounding the spore become more marked, while the staining of the soma and of the remaining portion of the capsule recedes. The steps in this process are shown in Figs. 5, 6 and 7. (See also Fig. 12). Finally a stage is reached in which only a purplish-black horseshoe is seen which may be attached to the ghost of the former vegetative body, or may lie entirely alone, all other trace of the original organism having disappeared. When the organism has completely sporulated, the hyaline unstained spores are seen surrounded by a line (no longer thick and black) of blue-black material which may be closed to form a circle, but is more often open in one small segment, like the original horseshoe which it appears to have succeeded.

If the organism, when sporulation is well under way, be stained by the Burke method for spores[4], both spore and horseshoe are not infrequently stained. The pink spore is to be seen snugly embraced by the horseshoe. It remains in this position until the process of

[4]Burke, G. S. Differential stain for living and dead spores. J. Inf. Dis., **23**, 433. 1923.

sporulation is complete. I have never seen it being expelled (as an egg might be laid) as described by Neumann.[5] This process, if it ever occurs, must be extremely rare; otherwise it would have been encountered in the hundreds of films we have examined.



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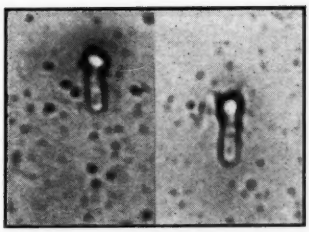

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Fig. 10. Six individuals from a specimen of *Cl. tetani* showing the clearly marked capsule. Notice the thickening of capsular membrane in the sporulating individual.

Fig. 11. Seven individuals from a specimen of *Cl. Welchii*. Notice that, save for the greater thickness of the soma in some of the forms, the picture is quite similar to that of *Cl. tetani*. (See also Fig. 9).

Fig. 12. Two individuals from a specimen of *Cl. tetani* during sporulation. Note the horseshoes about the spores and the recession of the rest of the bacterial somata.

It is not to be understood that the validity of this process of sporulation has been established by observing the actual passage of one form into another. The process has been reconstructed by

[5]Neumann, Harry. Die Isolierung und die Entwicklung von Anaërobiersporen. Centbl. Bakt. I Abt., **115**, 100. 1930.

observing the appearance in young cultures as shown in Fig. 1, and in old cultures as shown in Fig. 7. The other forms described have been met in successive examinations made of the cultures from the time when they consist entirely of vegetative forms to the time when they are made up entirely of spores. It seems probable, therefore, that the chronology of the process is correctly represented in the illustrations.

The difficulty referred to in a previous publication[6] presented by the accumulation of stains at the so-called "capsular membrane", presents itself also in the staining of the striking horseshoe-like structure formed about the spore during its development. It is not easy to understand why this blue-black horseshoe is so clearly visible at the periphery, but is hardly, if at all, to be made out over the surface of of the spore as one focuses up and down.

The noteworthy changes described are observed, not only when sporulation takes place in organisms growing on a culture medium, but also when the vegetative forms are allowed to sporulate in suspension in sterile water.

[6]See footnote 3.

## NOTES ON TECHNIC

METHOD FOR SIMULTANEOUS DEMONSTRATION OF STARCH AND FAT IN PROTOZOA.—Under some circumstances it is highly desirable to stain the starch and fat in protozoa simultaneously. Working with the flagellate, *Chilomonas paramecium*, the following method proved successful: Two stock solutions are prepared, one containing a 0.3% solution of iodine in 60% alcohol, the other a saturated solution of sudan III or scarlet R in a mixture containing 60% of alcohol and 10% of acetone. Equal parts of each solution are mixed in a small bottle to contain 24 hours' supply. The stock solutions keep indefinitely, whereas the mixture decomposes in 36 to 72 hours. The amount of acetone may be varied as the solubility of the fat permits. The higher the percentage of acetone, the more intense the fat stain. The mixture may be used as a killing fluid or the organisms may previously be killed with neutral saline formol.—W. L. DOYLE, Zoological Lab., Johns Hopkins University.

A NOTE ON IRON HEMATOXYLIN.—Shortly after the publication of a recent note on improving the staining action of iron hematoxylin (see abstract below, p. 119) one of the advocated procedures began to give trouble. The stain, altho freshly made from the stock 10% alcoholic solution, failed to differentiate sharply and the cytoplasm remained a muddy brown. After eliminating all other factors, it was found that the stock alcoholic solution had deteriorated despite the lack of visible evidence. The stock solution was three years old. Preparation of a fresh stock solution corrected all difficulties.

These experiences would seem to indicate that an alcoholic stock solution of hematoxylin has a limited period of usefulness. For complete assurance of perfect staining, it would be better to make a fresh stock solution once a year. The 0.5% solution that is used for staining will not deteriorate in less than three months. When a trace of sodium bicarbonate is added to a new 0.5% solution it may be used at once and reaches maximum effectiveness within a few hours.—ROBERT T. HANCE, University of Pittsburgh.

## BOOK REVIEW

BAKER, JOHN R. Cytological Technique. xii + 131 pp. Methuen & Co., London, 1933. 3s. 6d.

Out of numerous formulae and methods scattered in the literature, the author describes only those deserving application according to his own opinion and experience. The spirit of this compact, small book is illustrated in the following words of the author: "It is the purpose of this book to describe methods of preserving cells or parts of cells in a life-like way, and to explain them, so far as possible, with our present knowledge, in terms of chemistry."

Of the 121 pages of text, 80 (4 chapters) are taken up by detailed discussion of the physical and chemical action of simple and compound fixatives on the animal cell and its components. Besides simple preservation, these chapters deal with questions of precipitation, artefacts, rate of penetration, degree of shrinkage, and effect on the physical properties of the tissue.

High osmotic pressure, contrary to the idea of those investigators who make up formaldehyde or other substances with isotonic saline, has no direct shrinking effect, or *vice versa*. Five per cent acetic acid swells tissue, altho it has a pressure about 3 times that of the fluid of mammalian cells. Saturated picric acid shrinks cells, altho its pressure is only one-third that of the cells. Picric acid is a strong precipitant of proteins of the cytoplasm; while acetic acid causes no precipitation. This knowledge will prevent ill-considered methods and will help the investigator to choose or to invent fixatives suitable for his material.

In Chapter V, embedding is discussed. Chapters VI and VII are taken up with the subject of stains and staining. A brief, but very clearly expressed, discussion is given therein of the chemistry of dyes and of the theory of staining.

Finally, a short discussion on mounting is given in Chapter VIII

## LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked. Thru coöperation with the Wistar Institute of Anatomy and Biology of Philadelphia, these same abstracts are to appear on the Laboratory Service Cards of that institute. Those desiring some of these cards without subscribing for the entire Bibliographic Service of the Wistar Institute, can order from STAIN TECHNOLOGY, at a price of 6 cents each.

BURGHGREAVE, P. Etude sur la valeur comparative de différents fixateurs en vue de la recherche du glycogène dans les tissus. Bull. d'Hist. Appl., **10**, 73–86. 1933.

The effects of various fixatives, methods of embedding, staining, etc. on the presentation of glycogen in the liver of the white rat were observed. The author's conclusions are:

Commercial formalin, saturated with dextrose, is an excellent fixative for glycogen, but hardens the tissue. Absolute alcohol is a good precipitant, but being a slow penetrant, it does not prevent glycolysis in the inner portions of the material. Bark's absolute alcohol-formalin, or Vastarini-Cresi's fixative (95% alcohol, 100 pts.; formalin, 10 pts.; acetic acid, 5 pts.) are very satisfactory. The latter fixative preserves the tissue without rendering it brittle. Contrary to Best's opinion, the author found that paraffin embedding is in no way inferior to that of celloidin. A 5% celloidin solution may be brushed over the block prior to every cut, if the material is hard. The application of water instead of alcohol for the sections has no influence on glycogen.

Best's carmin for glycogen is the preferred. Staining for 30 to 60 minutes is recommended.

HANCE, R. T. Improving the staining action of iron haematoxylin. Science, **77**, 287. 1933.

The author finds that sodium bicarbonate has an excellent effect on the staining quality of iron hematoxylin. Prepare a 10% solution of hematoxylin in 95% alcohol for the stock solution (recommended in Kingsbury and Johannsen's "Histological Technique"), which keeps indefinitely. Dilute 5 cc. of the stock in 100 cc. water, and add a very small quantity of sodium bicarbonate, (as much as can be lifted on the point of a scalpel) to 1 liter of the solution.

This procedure has been successfully used in the author's and neighboring laboratories for 3 years. The solution keeps for about 6 months or longer and stains well as long as it remains clear. Grübler's and MacAndrew & Forbes' hematoxylins have been used. (See note on p. 117 of this issue for a later modification of the technic.)

KATO, KATSUJI. A method of preparing blood smears. J. Lab. & Clin. Med., **18**, 527–529. 1933.

Altho of great importance in hematologic studies, the proper spread of blood smears has received very little attention in literature. The two most commonly employed methods are Ehrlich's two-cover-glass method, and the two-slide method. The first is cumbersome in handling, and by means of the other leucocytes are greatly distorted or pushed to the edges.

The method here described is that used by Dr. W. Bloom, of the Department of Anatomy, University of Chicago. Deposit a drop of blood, about 3 to 5 mm. in diameter, from a fresh stab wound on a slide slightly away from the center. Cover immediately crosswise with a cover glass 24 to 50 mm. and allow the drop to spread without exerting pressure. After the drop has spread to a circle of appropriate dimensions draw the cover quickly across towards the other end of the slide. Fan the slide at once and air dry.

This should give an elongated oval of thin film, the border of which should be well within the edges of the slide. Leucocytes should be evenly distributed in almost every field. The thickness of the film may be controlled by the length of time allowed to lapse from the moment the cover glass is applied.

Each worker will have to learn the finer details and particulars from personal experience and to suit the blood of the various animals.

KOERTH, C. J. & HIBBARD, R. J. B. The comparative efficiency of the three stains for tubercle bacilli. Lab. & Clin. Med., **18**, 535–537. 1933.

Sputa of 600 patients were stained by each of the following three methods. 1) Ziehl-Neelsen: Steam with carbol fuchsin 5 min., wash, acid alcohol 8 min., wash, 1% methylene blue 30 sec. 2) Schulte-Tigges: Steam with carbol fuchsin 1 min., wash, decolorize with 10% sodium sulfite until color disappears, wash, counterstain with saturated picric acid, wash, dry. 3) Spengler: Stain with carbol fuchsin (warm, but not hot enough to bubble) 5 minutes, pour off stain and add picric acid alcohol for 4 to 5 sec. (1 part saturated solution aqueous picric acid to 1 part 95% alcohol), wash with 60% alcohol, treat with 15% nitric acid until yellow (not over 30 seconds); counterstain with picric acid alcohol until lemon yellow. Wash with distilled water and dry.

Ten fields of each case were examined making a total of 18,000 counts. The total number of bacteria counted following the first method were 19,692; following the second, 51,759; and the third, 71,559. There were 2087 negative fields with the first, 1577 with the second, and 1504 with the third.

Schulte-Tigges' method is only slightly less efficient than Spengler's. It is much more difficult to perform, however, and the chance of error in technic is large. The sodium sulfite must be absolutely fresh. Spengler's method takes only 7 minutes, while Ziehl-Neelsen's, as used in this particular laboratory, requires 15. The former is, therefore, preferred.

SEKI, KASAJI. Zur physikalischen Chemie der histologischen Färbung. III. Über die Karminfärbung. Folia Anatomica Japonica, **11**, 1–13. 1933.

(The first two papers of this series were reviewed in Stain Techn. **8**, 80, 1933.)

The isoelectric point of carmin, at which the dye is of minimum electrical charge and is practically insoluble, lies at about pH 4.0 to 4.5. In an alkaline or strongly acid reaction it is soluble because it has become positively or negatively charged respectively.

Carmin is positively charged by the presence of alum or aluminium sulfite, and goes into solution.

When carmin is positively charged with hydrogen-ions or aluminium-ions it stains like a basic dye; i. e., it dyes the negatively charged silicon dioxide and it progressively stains, moreover, the strongly negatively charged cell nucleus, the hyaline cartilage, and mucus. In contrast to this, the negatively charged carmin does not color silicon dioxide. In an alkaline solution it stains the tissues only diffusely, as they are in general negatively charged; and it is only after the subsequent differentiation with strongly acid solutions that the nuclei, etc. stain with the now positively charged carmin, while the background decolorizes.

SEKI, KASAJI. Zur physikalischen Chemie der histologischen Färbung. IV. Über die Hamateinfärbung. Folia Anatomica Japonica, **11**, 15–36. 1933.

Hematoxylin acts as a weakly negatively charged dye; its oxidation products, hematein and oxyhematein, bear a strongly negative charge. The isoelectric point of hematein is about pH 6.5.

When a hematoxylin solution is allowed to ripen, the dye becomes more negative, and can, therefore, more readily combine with alum or iron-alum to form a lake. The lake thus produced has a strongly positive charge and stains like a basic analin dye, i. e., coloring the nuclei deeply and the cytoplasm and collagen fibers more weakly. Hematein charged with positive hydrogen-ions also stains like a basic dye without being converted into a violet lake.

The hematein lake stains like a basic dye merely because it is positively charged and not because it is changed into a violet, easily flocculated color.